

Methanogenic and Other Strictly Anaerobic Bacteria in Desert Soil and Other Oxic Soils

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Strictly anaerobic bacteria such as methanogenic, sulfate-reducing, and homoacetogenic bacteria could be enriched from all five oxic soils tested. The number of cells was lower than that in typical anoxic habitats. Spores did not always dominate the population of sulfate-reducing bacteria. In all soils, the methanogenic population displayed a long lag phase after anoxic conditions were imposed before methane production began.

Methanogenic, sulfate-reducing, and homoacetogenic bacteria, normally considered to be strict anaerobes, have been isolated mainly from typical anoxic habitats. However, strictly anaerobic bacteria can be found in low numbers even in well-aerated, i.e., oxic, soils. Mayer and Conrad (23) showed that the methanogenic population in rice paddies stays constant during dry fallow periods, and even in forest and arable soils, a very small methanogenic population which becomes active under anoxic conditions and produces methane exists (23). Other results show that methanogenic bacteria can survive incubation in an oxygen-containing atmosphere (13, 18).

Canfield and DesMarais (7) and Fründ and Cohen (14) showed that aerobic microbial mats contain sulfate-reducing bacteria, which were also active under oxic conditions. Oxygen-protecting enzymes such as superoxide dismutase and catalase have been found in sulfate-reducing bacteria (16, 17) and were proposed to explain the survival of the bacteria after exposure to oxygen. Also, superoxide dismutase activity has been found in some methanogenic bacteria (19, 20). Cypionka et al. (11) discovered that sulfate-reducing bacteria can survive short exposure to oxic conditions, and later experiments indicated that 14 species of sulfate-reducing bacteria tested were even able to carry out aerobic respiration (12). Anaerobic microniches, for example those caused by oxygen usage by facultative anaerobes (15), might also explain the ability of anaerobic bacteria to survive under apparently oxic conditions.

These findings raise the question of whether the presence of strictly anaerobic bacteria in oxic habitats is an exceptional occurrence or more common than supposed. We tested the following five different oxic soils for the presence of methanogenic, sulfate-reducing, and acetogenic bacteria: cultivated soil (Marburg, Germany [27]), forest soil (Marburg, Germany [26]), termite soil from fungus-cultivating termites (Lamto, Ivory Coast [2]), savanna soil (Lamto, Ivory Coast [1, 3, 6]), and desert soil (Karoo, South Africa [9]). The main criteria for selecting these soils were well-aerated soil status (i.e., oxic soils) and the absence of a history of methanogenesis at these sites (except for the termite soil). The soils thus did not provide suitable habitats for microorganisms with anaerobic metabolism. The main characteristics of these soils are listed in Table 1. The maximum water-holding capacity, the organic carbon content, and the actual soil moisture of the soils (Table 1) were determined by standard techniques (28). In all cases soil sam-

ples were taken from the A horizon (top 10 cm). Sampling was carried out when the soils were in a dry condition (0 to 33% water-holding capacity). The soil samples were placed in polyethylene bottles and stored under air at 4°C (with the exception of the desert soil, which had been stored at room temperature since 1983). Table 1 shows the dates of sampling and the storage time. Before use, the soils were passed through a sieve (2-mm mesh). Sterile anoxic water was added at a ratio of 1:1 (wt/vol) to prepare slurries.

A bicarbonate-buffered mineral medium (30) at pH 7.0 under a gas phase of N₂-CO₂ (88%:12%) was used for cultivation. The medium was supplemented with vitamins, trace elements, and 1 mM sulfide as a reducing agent (30). Different substrates (Table 2) were added to enrich specifically methanogenic, sulfate-reducing, or homoacetogenic bacteria. Inoculations of 2 ml of slurry were incubated for 2 months at 30°C. Enrichments were checked microscopically and were scored positive when methane production (determined by gas chromatography [8]) exceeded 0.03 µmol/g of soil, when the acetate concentration (determined by high-pressure liquid chromatography [21]) exceeded 500 µM, or when a definite browning of the sulfide-testing solution (10) was observed; other results were counted as negative.

The results of the enrichments (Table 2) clearly demonstrated the presence of strictly anaerobic bacteria in all five oxic soils. Not only the cultivated, forest, and termite soils but also the savanna and desert soils contained methanogenic, sulfate-reducing, and homoacetogenic bacteria. The enrichment and counting conditions such as temperature, pH value, and choice of trace elements and vitamins were standardized for all soils tested. The enriched and counted bacteria thus probably represent only a fraction of the actual microfloras in the soils. Concerning this fact, the number of cells and the variety of microorganisms found are only conservative estimates.

The length of storage was different for the various soils but had no obvious influence on the results. A shift in the bacterial population was not expected because of the low water content, the low temperature (4°C), and the oxic conditions during storage. Indeed, the sulfate content of the stored soils remained constant from the beginning (April 1992) until the end (February 1993) of our study, indicating the absence of any bacterial sulfate reduction. Also, the potential methane production rates that were inducible under anoxic conditions (see below) were reproducible at any time (three repetitions) during this period. Finally, replication of the enumeration of methanogens in the soils after an additional storage time of 6

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TABLE 1. Characteristics of soils tested

Soil	Location	Type	Water-holding capacity (g of H ₂ O/100 g [dry wt] of soil)	pH	Organic C (%)	Sampling date	Avg temp at sampling (°C)	Actual soil moisture (g of H ₂ O/ 100 g [dry wt] of soil)	Storage	
									Time (months)	Temp (°C)
Cultivated (cereals)	Ronhausen, Marburg, Germany	Loam, dystic cambisol	37	6.3	1.9	October 1991	10	3.1	7	4
Forest (<i>Fagus sylvatica</i>)	Lahnberge, Marburg, Germany	Clay, orthic luvisol	76	3.9	7.5	April 1992	10	24.9	0	4
Savanna (<i>Hyphantia</i> sp.)	Lamto, Ivory Coast, Africa	Loamy sand, ferralsol	33	7.1	2.05	February 1992	27	0	2	4
Desert	Karoo, South Africa	Sand, sierozem	23	7.5	1.25	February 1983	25	0.13	110	RT ^a
Termite (<i>Ancis termites</i>)	Lamto, Ivory Coast, Africa	ND ^b	ND	ND	ND	February 1992	27	ND	2	4

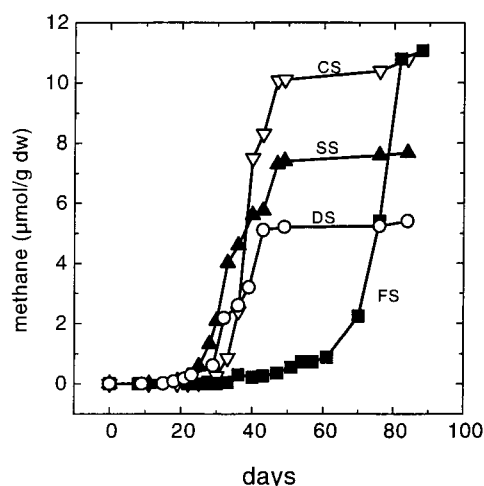
^a RT, room temperature.^b ND, not determined.

FIG. 1. Methane production by anoxic slurries (20 g [dry weight] of soil and 20 ml of sterile water, with no added substrate) of four oxic soils: cultivated soil (CS), forest soil (FS), savanna soil (SS), and desert soil (DS). The data are the means for triplicate measurements. The coefficient of variation was <14.8% dw, dry weight.

months resulted in similar numbers (deviation factor of <5.8%).

The detection of methanogenic bacteria was especially surprising, because in contrast to sulfate-reducing or homoacetogenic bacteria, there are no spore-forming or other known resistant species. On all substrates tested (Table 2) and in all soils, methanogenic bacteria were detected. It was striking that in all methanogenic enrichment cultures, coccus-shaped bacteria dominated, a finding which leads to the assumption that the methanogenic population mainly consists of the metabolically versatile methanogenic bacterium *Methanosarcina* sp. This result indicates that the dry, oxic conditions in situ and also during storage were especially favorable for the survival of coccus-shaped bacteria, e.g., *Methanosarcina* spp. Alternatively, the dominance of these bacteria may be due to our specific enrichment conditions.

The number of bacteria were ascertained by the most probable number method (29). Serial dilutions (10-fold) of the soil slurry were performed in triplicate with mineral growth medium. The inoculum (1 ml) was vortexed into 9 ml of medium at the next higher dilution step. However, because sufficient separation of microaggregates of bacterial consortia cannot be confirmed, the most probable number method may have underestimated the actual number of bacteria.

The most probable number of methanogenic bacteria able to utilize H₂ and CO₂ varied between 36 and 9,600 cells per g (dry weight) of soil (Table 3). The highest numbers were found in the cultivated and termite soils, with 9.6×10^3 and 6.0×10^3 cells per g (dry weight), respectively. The data were confirmed by enumeration of replicate soil samples.

The low number found show that the population of methanogenic bacteria is smaller than that in typically or partly anoxic habitats: the most probable number of hydrogenotrophic methanogenic bacteria in rice paddies was 10^5 to 10^6 cells per g (dry weight) during the entire growing season (23).

Sulfate-reducing bacteria were detected in all five soils tested. Enrichments of the cultivated soil were positive for five of six substrates tested, whereas the termite and desert soils were positive for only two substrates each (Table 2). Only the substrate H₂-CO₂ (88%:12%)-1 mM acetate-10 mM sulfate produced positive enrichment results for all soils (Table 2).

TABLE 2. Survey of methanogenic, sulfate-reducing, and acetogenic bacteria cultivated from five soils tested

Bacteria	Substrate	Growth in soil sample indicated				
		Cultivated	Forest	Savanna	Desert	Termite
Methanogenic	H ₂ + CO ₂ ^a	+	+	+	+	+
	10 mM methanol	+	+	+	+	+
	20 mM acetate	+	+	+	+	+
Sulfate reducing	H ₂ + CO ₂ ^a + 1 mM acetate ^b	+	+	+	+	+
	20 mM acetate ^c	+	—	—	—	+
	10 mM propionate ^b	+	—	—	—	—
	10 mM butyrate ^b	—	+	+	—	—
	10 mM lactate ^b	+	+	+	—	—
	1 mM benzoate ^b	+	+	—	+	—
Homoacetogenic	1 mM gallic acid	+	+	+	+	+
	1 mM syringic acid	+	+	+	+	+
	1 mM trimethoxybenzoic acid	+	+	+	+	+
	H ₂ + CO ₂ ^a + 0.1 mM CHCl ₃	+	+	+	+	+

^a H₂ + CO₂ (88%:12% [vol/vol])^b Supplemented with 10 mM Na₂SO₄.^c Supplemented with 20 mM Na₂SO₄.

The numbers of sulfate-reducing bacteria able to utilize this substrate were determined. It was striking that the sulfate-reducing population in the cultivated soil (1.84×10^4 cells per g [dry weight]) was much larger than those in the other soils (Table 3). The cultivated soil showed a concentration of sulfate (0.23 mM; determined by ion chromatography [5]) that was about 3- to 100-fold higher than those in the other soils (2.5 to 70 μ M). This might explain the higher number of sulfate-reducing bacteria in the cultivated soil.

To see whether spore-forming sulfate-reducing bacteria were the major part of the population, the soils were pasteurized to determine the number of sulfate-reducing bacteria present as spores. Formation of spores might be a strategy of survival under oxic or partly oxic conditions. Particularly in the cultivated soil, the low number of sulfate-reducing bacteria detected after pasteurization (Table 3) shows that most of the H₂-CO₂-utilizing population of sulfate reducers was not present as spores and obviously used another strategy to survive oxic conditions. Their ability to carry out at least limited aerobic respiration (11, 12) may be important in this respect. The percentage of spores in the population of acetate-utilizing sulfate reducers, e.g., spore-forming *Desulfotomaculum* spp., was not determined, since this metabolic type was only found in enrichment cultures from the cultivated and termite soils.

In all five soils tested, and with four different substrates, it was possible to enrich acetate-producing bacteria, which presumably were homoacetogens (Table 2). This conclusion is

based on the use of aromatic compounds that are typically used for enrichments of homoacetogens (4). Furthermore, enrichments were also successful on H₂-CO₂ in the absence of sulfate (supporting sulfate-reducing bacteria) and in the presence of chloroform (inhibiting methanogenic bacteria).

The forest soil, with 6×10^4 cells per g (dry weight), contained the highest number of homoacetogenic bacteria able to utilize H₂-CO₂ (88%:12%). Chloroform was added to inhibit methanogenic bacteria. Aerotolerance of the homoacetogenic bacteria has, to date, not been investigated. The measured acetate production on aromatic compounds (Table 2) also suggests the presence of homoacetogenic bacteria. However, since homoacetogenic bacteria are not the only organisms that can form acetate from aromatic compounds, we cannot be sure that acetate was produced exclusively by homoacetogenic bacteria. However, the existence of homoacetogenic bacteria in forest soil was shown recently by Küsel and Drake (22). Our study confirms and extends this observation.

To test the time required for methanogenic bacteria to become active under anoxic conditions, 20 ml of sterile water was added to 20 g of soil in 120-ml serum vials. Incubation was carried out at 25°C under an N₂ atmosphere, and methane production was measured over a period of 2 months. The experiments were repeated twice, 2 and 6 months later, and gave almost identical results. Significant methane production (>6 nmol/g [dry weight] of soil) was first observed in the desert soil after 15 days, in the savanna soil after 19 days, in the

TABLE 3. Bacterial populations in soil samples tested

Soil	No. of bacteria ^a			
	Methanogenic	Sulfate reducing		Homoacetogenic
		Nonpasteurized	Pasteurized ^b	
Cultivated	9,600 (1,440–52,000)	18,400 (2,840–96,000)	186 (30–700)	4,400 (600–19,200)
Forest	186 (30–760)	36 (4–144)	86 (14–420)	60,000 (12×10^4 – 17.6×10^6)
Savanna	36 (4–188)	172 (28–840)	18 (2–72)	36 (4–144)
Desert	36 (4–188)	36 (4–144)	14 (2–46)	4,400 (600–19,200)
Termite	6,000 (1,200–17,600)	372 (60–1,520)	186 (30–760)	300 (60–880)

^a Number (cells per gram [dry weight] of soil) of bacteria able to utilize H₂-CO₂ (88%:12% [vol/vol]) alone (methanogenic and homoacetogenic bacteria) or supplemented with 1 mM acetate and 10 mM sulfate (sulfate-reducing bacteria). The 95% confidence intervals are given in parentheses.

^b Pasteurized for 30 min at 80°C.

cultivated soil after 22 days, and finally in the forest soil after 30 days of anoxic incubation (Fig. 1). One explanation for such a long incubation time before the onset of production might be that energetically more-favorable (e.g., nitrate or sulfate reduction) redox processes were more competitive than the methanogens (25), or perhaps the very small population of methanogenic bacteria (Table 3) needed this time to grow. In any case, acetate was the main precursor of CH₄ and was produced in relatively large concentrations (>2 mM) prior to the onset of CH₄ production (24).

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